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Mechanical characterization of regenerating *Hydra* tissue spheres

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33 **Abstract**

34 *Hydra vulgaris*, long known for its remarkable regenerative capabilities, is also a longstanding source
35 of inspiration for models of spontaneous patterning. Recently, it became clear that early patterning
36 during *Hydra* regeneration is an integrated mechano-chemical process where morphogen dynamics is
37 influenced by tissue mechanics. One roadblock to understand *Hydra* self-organization is our lack of
38 knowledge about the mechanical properties of these organisms. In this paper, we combined
39 microfluidic developments to perform parallelized microaspiration rheological experiments and
40 numerical simulations to characterize these mechanical properties. We found three different
41 behaviors depending on the applied stresses: an elastic response, a visco-elastic one and tissue
42 rupture. Using models of deformable shells, we quantify their Young's modulus, shear viscosity as well
43 as the critical stresses required to switch between behaviors. Based on these experimental results, we
44 propose a description of the tissue mechanics during normal regeneration. Our results provide a first
45 step towards the development of original mechano-chemical models of patterning grounded in
46 quantitative, experimental data.

47 **Statement of significance**

48 *Hydra vulgaris* is a remarkable organism thanks to its regenerative abilities. One can cut this animal
49 into several pieces which will reform a full *Hydra* in a few days. In this process, the pieces have to
50 define a new organizing axis. Recently, researchers have shown that this axis definition is under
51 mechanical control. One roadblock to understand the relationship between tissue mechanics and
52 *Hydra* biology is our lack of knowledge about the mechanical state of this organism. Here, we perform
53 a mechanical characterization using a combination of microaspiration setups and numerical
54 simulations. We finally propose a description of what happens at the mechanical level during *Hydra*
55 regeneration, allowing quantitative approaches questioning the role of mechanical cues in axis
56 definition.

57

58 **Introduction**

59 *Hydra vulgaris* has long been a model of choice in developmental biology because of its remarkable
60 regenerative capabilities (1, 2). Almost any excised tissue piece as well as cellular re-aggregates are
61 capable of reforming a fully viable adult in just a few days. In the former example, an excised tissue
62 piece folds back into a closed spherical shape with both its epithelial monolayers, endoderm and
63 ectoderm, engulfing a water-filled lumen. In the latter, the same tissues start by spontaneously sorting
64 into their relative positions (3, 4) before expelling excess cells to reform a hollow sphere. At this point,
65 both regenerative trajectories converge and these tissue spheres start undergoing osmotically-driven
66 oscillations during which they swell because of water flowing from the environment to the lumen up
67 to the point where the tension building up within the tissues becomes too large and leads to a local
68 rupture (5, 6). After rupture, the samples deflate, close the resulting wound and start another swelling-
69 rupture cycle. These osmotic oscillations induce deformations and therefore stresses within the tissues
70 with relative changes in the radii of the spheres up to 30%. These have been referred to as phase I
71 oscillations and are characterized by a high amplitude and a low frequency (6). Indeed, after a few of
72 these cycles, the oscillations clearly change and enter phase II where they have lower amplitude and
73 higher frequency (6).

74 In parallel to these mechanical oscillations, the samples establish a chemical pattern involving some
75 characterized morphogens to define the oral-aboral axis of the organism. Most notably, a local
76 expression of HyWnt3 has been shown to be an early signal of axial patterning with this activation

77 defining the future position of the head organizer (7). This local activation is then followed by the
78 establishment of various chemical gradients within the spheres effectively patterning the whole axis
79 (8, 9). At that point, the originally symmetrical samples start elongating in an oblong shape at which
80 ends the adult organs of the head and foot will be regenerated, and the patterning can be considered
81 complete. The switch between phase I and phase II oscillations was thought to be a signature of the
82 establishment of axial patterning (6, 10). Since small excised tissue pieces showed both phases, it was
83 thought that they underwent spontaneous symmetry breaking and retained no memory of axial
84 patterning just as cellular re-aggregates (5, 6).

85 The question was then to determine how the spherical symmetry was broken during phase I
86 oscillations and how the local head organizer was defined. For a long time, the main hypothesis was
87 that of a purely biochemical spontaneous symmetry breaking in the form of Turing-instabilities of an
88 unknown reaction-diffusion system. In his seminal work, partially inspired by *Hydra* regeneration,
89 Turing has shown how a system of two interacting and diffusing chemical species, which he named
90 morphogens, could become unstable in their homogenous state and spontaneously start forming
91 structures such as dots or stripes (11). These ideas were critical in developing the field of pattern
92 formation and have been adapted to a wide variety of systems and organisms (see for example (12,
93 13) for recent reviews in the context of developmental biology). In this historical development, *Hydra*
94 has retained its place as a model organism. Notably, Gierer and Meinhardt have expanded on the
95 seminal ideas of Turing and developed a modified version of his reaction-diffusion system specifically
96 designed to explain regeneration and grafting experiments in *Hydra* (14). As a result, the so-called
97 Gierer-Meinhardt model has long been the gold standard in the field. At its inception, it was purely
98 speculative but, since then, some proteins have been shown to possess many of the characteristics
99 required by the Gierer-Meinhardt model. Most notably, the protein HyWnt3, involved in the canonical
100 Wnt pathway is now generally considered to be the activator represented in the Gierer-Meinhardt
101 model (15) since its expression is restricted to the head organizer, it has self-activating capacities and
102 it is the first temporal signature of symmetry breaking during regeneration. However, the necessary
103 long-range inhibitor is notoriously missing. Some promising candidates were put forward, such as
104 Dickkopf (Dkk) (16), Sp5 (17) or *Hydra* astacin-7 (18) but none of these could reproduce the predictions
105 of these models, mostly because of differences in expression patterns.

106 These models also ignore the mechanical aspects of the process, most notably the osmotic oscillations
107 although it is now well established that they are necessary for proper regeneration (19, 20). This
108 observation suggested a possible coupling between the mechanical state of the tissues and their
109 biological response, as has been observed in a variety of contexts and organisms with impacts on cell
110 division or gene expression including in the canonical Wnt pathway (21–24). Recent results have
111 demonstrated such a coupling in *Hydra* by which the expression of HyWnt3 is reduced when osmotic
112 oscillations are blocked (20) providing a potential direct coupling between tissue mechanics and
113 chemical patterning.

114 Recent modelling efforts have thus been made in order to incorporate mechano-chemical couplings
115 (19, 25, 26), for instance by making the diffusion constant of the morphogens within the tissues
116 dependent on tissue stretch (19). In most cases though, these couplings were not grounded in
117 experimental evidence. In addition, recent experimental results have started to question the
118 assumptions described so far and underlying these models. First, it was shown that the shift from
119 phase I to phase II oscillations was not a direct signature of axial patterning being established. Instead,
120 the onset of phase II is due to the early apparition of the *Hydra* mouth (27) allowing it to regulate its
121 osmotic imbalance by mouth opening rather than by tissue rupture. One consequence was that axial
122 patterning had to be anterior to the shift between oscillation phases. It then became unclear whether

123 small, excised tissue pieces really went through *de novo* patterning or if they could inherit this
124 patterning from their original host organism. It now appears that they do retain axial patterning
125 although the exact mechanism by which they do so remains unclear, whether by the organization of
126 their ectodermal actin structures called myonemes (28) or by pre-existing biochemical gradients (29).

127 Although small excised tissue pieces retain axial patterning, it still remains that 1- cellular re-aggregates
128 which cannot conserve either supracellular actin structures or chemical gradients have to show *de*
129 *novo* axial patterning during osmotic oscillations, 2- there is evidence of a direct coupling between
130 tissue deformations and Hywnt3 expression (20), one of the most important morphogens involved in
131 *Hydra* patterning and 3- osmotic oscillations are required for the proper elongation, morphogenesis
132 and regeneration of both excised tissue pieces and cellular re-aggregates. For all these reasons, the
133 focus of the field is currently shifting to an integrated view of *Hydra* regeneration as a mechano-
134 biological process (26, 30). One clear roadblock to the development of these ideas is our lack of
135 understanding of the rheological properties and mechanical state of regenerating *Hydra* tissue
136 spheres. This often leads to assumptions as to the rheology of these samples and rough estimates of
137 their key mechanical parameters. One exception is the development by Veschgini and colleagues of a
138 two-fingered robotic hand allowing them to apply known, constant deformations on the tissue spheres
139 and measure the resulting forces (31) giving the first local, quantitative measurements of mechanical
140 features in *Hydra*. These measurements were not, however, used to deepen our understanding of the
141 spontaneous osmotic oscillations.

142 In this work, we try to overcome these limitations and to offer a quantitative characterization of the
143 mechanics of *Hydra* tissue spheres. To do so, we used the well-established micro-aspiration technique
144 (32–35) which we adapted to increase its throughput through the use of original microfluidic
145 constructs following (36). We found three different mechanical behaviors as the aspiration pressure
146 was increased: first an elastic response, then a viscoelastic one and finally tissue rupture, as observed
147 in phase I oscillations. Combining our experimental observations and measurements with the
148 development of a rheological model of elastic shells and numerical simulations, we obtained
149 quantitative measurements of both the main rheological parameters of *Hydra* tissue spheres and the
150 critical pressures required to switch between the three regimes. Thanks to these results, we provide a
151 description of internal tissue mechanics, strains and stresses during osmotic oscillations and reveal
152 that the tissue spheres behave largely as hyperelastic spherical shells during these oscillations.
153 Hopefully, this mechanical characterization will serve as a stepping stone for the study of mechano-
154 biochemical couplings in a quantitative manner.

155

156 **Material and Methods**

157 *Hydra* maintenance and lines used

158 3 *Hydra vulgaris* lines were maintained and used for experiments: a watermelon (WM) line
159 (ectoderm GFP / endoderm DsRed2), a reverse watermelon (RWM) one (ectoderm DsRed2 / endoderm
160 GFP) and the AEP line from which embryos were obtained for making transgenic animals. *Hydras* were
161 kept in *Hydra* medium (HM), which consists of 1mM CaCl₂, 0.1mM MgCl₂, 0.3mM KNO₃, 0.5mM
162 NaHCO₃ and 0.08mM MgSO₄ at a PH between 7 and 7.3. Cultures were stored at 18°C in the dark in
163 an incubator (Pol-Ekko-Aparatura). Animals were fed two to three times per week with newly hatched
164 Artemia (Hobby) and cleaned every two days by changing the medium in which they sit. Animals were
165 starved for at least 24h before the start of any experiment.

166 Preparation of tissue spheres

167 Tissue spheres were made by cutting a whole animal using a sterile scalpel (Holtex, Bistouri
168 UU n°10), as follows: The head and tail were removed by two transverse cuts and the remaining body
169 column was first sliced into 3 or 4 pieces. These pieces were cut further to obtain 6 to 8 samples per
170 adult animal. These samples were left to fold into tissue spheres for 3 to 4 hours in HM.

171 Design and fabrication of the removable inserts

172 Sliding elements containing the cylindrical pipettes were fabricated in dry film according to the
173 protocol detailed in (36). Briefly, photolithography was realized on a 500 µm-thick photosensitive dry
174 film (DF series), laminated on a silicon wafer. Lateral dimension of the photolithography mask defined
175 the length of the insert (20 mm) and its height (1 mm), as well as pipette diameter (50 and 100 µm
176 were used). 100 µm-wide square openings placed at the bottom of the insert were also used for
177 imaging purposes in Movie S3 and Fig S4. After post-exposure bake on a hot-plate, the inserts were
178 released from the wafer during overnight development in an acetone bath that both revealed the
179 patterns and unstick the dry films from the wafer. A single fabrication run allowed to fabricate 100
180 such sliding elements.

181 Mounting and preparing of the microfluidic device

182 We used a microfluidic tool that allows several micro aspirations to be performed in parallel.
183 This system is composed of a chamber crossed by a removable insert bearing multiple tunnels allowing
184 suction to happen. Both the chambers and inserts were designed using the CAD software Inventor
185 2020 (Autodesk). The chamber is composed of a main channel which is 20.5mm long, 4mm wide and
186 0.5mm high (see Fig S1 for the schematics). Halfway through its length, it is barred by another channel
187 which will host the removable insert. The perpendicular channel is 13mm long, 0.5mm wide and 1mm
188 high (Fig S1).

189 From this design, a mold was manufactured by micromilling using the CNC Mini-Mill/GX (Minitech
190 machinery corporation) with a two size cutter of 1mm diameter on a brass template. The chamber was
191 then made of polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) supplemented with 10% curing
192 agent (Dow Corning) from the mold. The PDMS was left to solidify overnight at 70°C and the channel
193 was manually peeled. Inlets and outlets were created by manual punching using a 1mm wide puncher
194 (Harris Uni-Core) at each end of the main channel. The insert channel was opened by manual,
195 transverse cutting to allow insertion of the sliding element. The resulting channel was then bound to
196 a thin PDMS layer, itself bound to a glass slide (Biosigma VBS655/A) by using an oxygen plasma cleaner
197 (Harrick plasma PDC002 with the associated PlasmaFlo gaz mixer). The thin PDMS layer was used to
198 facilitate the introduction of the insert by avoiding direct friction with the glass slide and to avoid
199 leakage. This introduction is done manually with tweezers and is aided by the fact that the insert
200 cannot deviate from its associated channel because of the height difference between both channels.
201 The outlet was connected to a plastic tube of 0.5mm internal diameter and 1.25mm external diameter,
202 sealed at the other end by a 0.6x30mm needle (BD, microlance 3) attached to a 3mL syringe (Braun,
203 Omnifix3P). The needle is deliberately slightly wider than the tube to ensure a hermetic connection.
204 The whole setup was placed in a 100mm Petri dish and immersed in HM. By slowly removing the
205 syringe's plunger, the channel started filling with HM from the inlet all the way to the syringe while
206 avoiding to create air bubbles in the main channel and checking for proper sealing of the system. Once
207 the plunger was fully removed, the height difference ΔH between the water in the syringe and the
208 water in the Petri dish (see Fig S2 for a schematic of the whole setup) was manually controlled and
209 created a pressure difference between chamber inlet and outlet according to:

$$210 \quad \Delta P = \rho g \Delta H$$

211 Where ρ is the HM density and g the gravitational acceleration.

212

213 Micro-aspiration experiments and data analysis

214 The syringe was first placed 4cm below the water level of the dish to create a light water flow induced
215 by hydrostatic pressure. Several *Hydra* tissue spheres were manually pipetted into the inlet and were
216 thus naturally driven to the holes in the inserts by the water flow, at which point the height difference
217 was set back to 0 for a couple of minutes. A typical experiment could then be started by the application
218 of a controlled pressure by moving the syringe down again. Images were captured under a microscope
219 (Zeiss Axio Vert.A1 equipped with an AxioCam 202 camera). In most experiments, timelapses were
220 recorded in fluorescent microscopy using WM and RWM lines.

221 In the case of elastic measurements, increasing steps of pressure were applied onto the same samples.
222 Each step lasted for 10min and images were recorded every 10s to make sure that no flow was
223 observed on the samples. After these 10min, the samples were released for 5min to allow them to
224 regain their original shape before applying a new, larger pressure difference.

225 For visco-elastic measurements, a single pressure step was applied and samples were imaged by
226 fluorescent microscopy for 30min with a 10s time step and the dynamics of the aspirated length was
227 monitored.

228 To automate the measurement of the aspirated length in these experiments, kymographs of the
229 aspirated tongue were generated in ImageJ (NIH), binarized and analyzed in Python with custom made
230 code to extract the length of the tongue in each image. For elastic measurements, the average length
231 over the last 5min of recording was used whereas for visco-elastic measurements, the whole dynamics
232 was kept.

233 The acquired images were also used to measure the mean projected area A of each analyzed sample
234 by usual thresholding techniques in ImageJ. This measurement was then turned into an average radius

235 R as $R = \sqrt{\frac{A}{\pi}}$.

236 Osmotic oscillations measurements

237 To measure osmotic oscillations shown in Fig S8, *Hydra* tissue spheres were prepared as described
238 above. In parallel, a 1% agarose gel was prepared at the bottom of a Petri dish, left to solidify and was
239 manually punched to create 1mm wide wells which then hosted the samples. These wells were used
240 to limit the samples movement during osmotic oscillations. They were then imaged for 24h at a 15min
241 interval. The resulting images were thresholded and analyzed in ImageJ to extract the projected area
242 as a function of time $A(t)$. Finally, the samples' radii $R(t)$ were computed as described above. The
243 equilibrium radius R_0 of a given sample was defined as the minimum of $R(t)$ over the whole recording
244 allowing us to estimate the strains on the inflating sphere.

245 Finite element simulations

246 Numerical simulations were performed with the finite-element solver Comsol Multiphysics (COMSOL,
247 Inc). The micropipette was represented as a rigid, motionless cylinder of inner radius $R_p = 50\mu\text{m}$. The
248 reference state of the *Hydra* sphere was a shell of outer radius $R_0 + h_0/2$ and inner radius $R_0 - h_0/2$,
249 whose center O was located on the cylinder's longitudinal symmetry axis Oz . We used contact
250 boundary conditions ensuring that the (deformed) *Hydra* sphere and the micropipette never overlap.
251 *Hydra* tissue was modeled as a hyperelastic Saint-Venant Kirchhoff material. We denote by E and ν
252 the Young's modulus and Poisson's ratio of the tissue and considered nearly incompressible conditions
253 ($\nu = 0.495$). The external pressure ΔP was applied on the spherical section contained within the
254 micropipette. Simulations were performed in 2D, enforcing the rotational invariance of the system

255 (*Hydra* + micropipette) about Oz . The equations of elasticity were treated within a pressure
256 formulation, adapted to approximately incompressible elastic materials. For each value of the applied
257 pressure, we recorded the maximal deformation observed on the symmetry axis denoted $\delta z(r = 0)$,
258 and computed as a spatial average along the shell's thickness. We checked that our results were robust
259 to smoothing the sharp corner of the micropipette by a fillet over a scale of the order of $5\mu m$. They
260 were also insensitive to decreasing the typical mesh size.

261 Extraction of rheological parameters

262 To measure Young's moduli in the elastic phase, each sample was treated separately. Based on the
263 acquired images, we measured their projected area S_0 and turned them into rest radii $R_0 = \sqrt{\frac{S_0}{\pi}}$. Then,
264 we used our measurements of ΔP as a function of δz (Eq 7) and fitted them by a straight line in Python.
265 The slope of this straight line was kept and multiplied, according to Eq 7, by $\frac{(1-\nu)R_0^2}{2C_S h_0}$ to extract the
266 Young's modulus of that specific sample. We then repeated the procedure on many samples and report
267 the mean and standard deviation of this distribution in the text.

268 In the visco-elastic phase, we measured the dynamics of the aspirated length as a function of time $L(t)$
269 and fitted them in Python according to Eq 8. To extract Young's moduli, we used the same formula as
270 above except that we replaced the slope of a linear fit of several points by the ratio of ΔP over $\delta(1 -$
271 $\kappa)$, δ and κ stemming from the fit of the full dynamics.

272 To estimate the order of magnitude of the effective viscosities in the same experiments, we used the
273 same fit according to Eq 8 to extract the speed of tissue flow U and estimated the viscosity as $\eta = \frac{\Delta P R_p}{U C_v}$
274 with $C_v = 1$.

275 Measurement and data analysis of threshold pressure measurements.

276 For the measurement of the pressure thresholds distinguishing each regime, we used the
277 fraction of pieces changing their rheological behavior at different applied pressures. To observe the
278 switch between the elastic and viscous regimes the pressure was gradually increased by lowering the
279 syringe in 3cm increments, starting low enough that no viscoelastic behavior was observed (usually 10
280 or 14cm). At each pressure level and after 20 minutes, we noted the fraction of pieces that had
281 changed regime, and the experiment was stopped when all samples had done so. Kymographs were
282 used to visually separate both regimes. The length profile over time was constant in the elastic regime,
283 whereas it evolved linearly with a clear slope in the viscous regime.

284 The same strategy was used to measure the rupture threshold, but we started at higher applied
285 pressures. Images were taken in transmission, as it facilitated the observation of briefly detached
286 pieces of tissue in the holes of the inserts, the first sign of the rupture regime. They were also taken
287 this time at a rate of ten minutes of suction per threshold. Identifying the switch between the viscous
288 and the rupture regime was done visually.

289 In both cases, we thus ended up with a fraction of samples adopting a certain behavior as a function
290 of applied pressure. Assuming that individual thresholds are variable and normally distributed, this
291 cumulative frequency followed a sigmoid shape of the form $\frac{1}{1+e^{-\frac{(\Delta P - \Delta P_c)}{\alpha}}}$ where ΔP_c is the threshold
292 pressure at which 50% of samples have switched behavior and α is a parameter controlling the
293 steepness of the sigmoid function. We thus fitted our data with that functional form using Matlab's
294 (Mathworks) curve fitting toolbox. In the manuscript, we report threshold pressures as ΔP_c resulting
295 from the fits and error bars represent the 95% confidence interval of these fits.

296 EDTA/blebbistatin experiments

327 Rheological measurements on *Hydra* whose cell adhesion had been inhibited were carried out by
328 immersing the tissue spheres in solutions of either 2 mM EDTA (Sigma-Aldrich) or 5 μ m blebbistatin
329 (Tocris Bioscience) in HM. The entire microfluidic system was immersed in the solution, and the *Hydra*
330 pieces were left to bathe in it for around ten minutes before aspiration to give the drug time to take
331 effect before measuring. The rest of the protocol was identical to that of the rheological measurements
332 explained above.

333 Spinning disk imaging

334 To estimate the thickness of *Hydra* tissue spheres, several WM samples were imaged underneath an
335 Eclipse Ti2 microscope (Nikon) equipped with a CSU-W1 spinning disk unit (Yokogawa) and an Orca
336 Fusion BT camera (Hamamatsu). Z-stacks were automatically acquired with a step of 1 micron and
337 vertical slices were made using ImageJ (Fig S4). The thickness of six samples were manually measured
338 using the same software and were all found to be in the 15 – 25 μ m range.

339 Mechanical description of *Hydra* tissue spheres

340 We consider a thin spherical shell of undeformed inner and outer radii $R_0 - \frac{h_0}{2}$ and $R_0 + \frac{h_0}{2}$. Normal
341 components of the Cauchy stress tensor are denoted σ_a ($a = 1,2,3$). Briefly, we re-express the strain
342 energy function, Eq 4, of a Saint-Venant Kirchhoff material as a function of the stretch ratios α_a :
343 $W(\bar{\mathbf{E}}) = \Psi(\{\alpha_a\})$. Denoting P the shell pressure field, stress components are given by $\sigma_a = -P +$
344 $\alpha_a \frac{\partial \Psi}{\partial \alpha_a}$. By the assumption of plane stress, $\sigma_3 = 0$, we obtain the expression of P and deduce the
345 circumferential, or hoop stress $\sigma = \sigma_1 = \sigma_2$. For an incompressible material, $4\pi R_0^2 h_0 = 4\pi R^2 h$,
346 where R and h denote the deformed radius and thickness, respectively. We note $\alpha = \alpha_1 = \alpha_2 = \frac{R}{R_0}$
347 the circumferential stretch ratios, and deduce the normal stretch ratio $\alpha_3 = \frac{h}{h_0} = \frac{1}{\alpha^2}$. The calculation
348 follows (37, 38) and yields the expression of the hoop stress of a Saint-Venant Kirchhoff spherical shell,
349 as given by Eq 11.

350

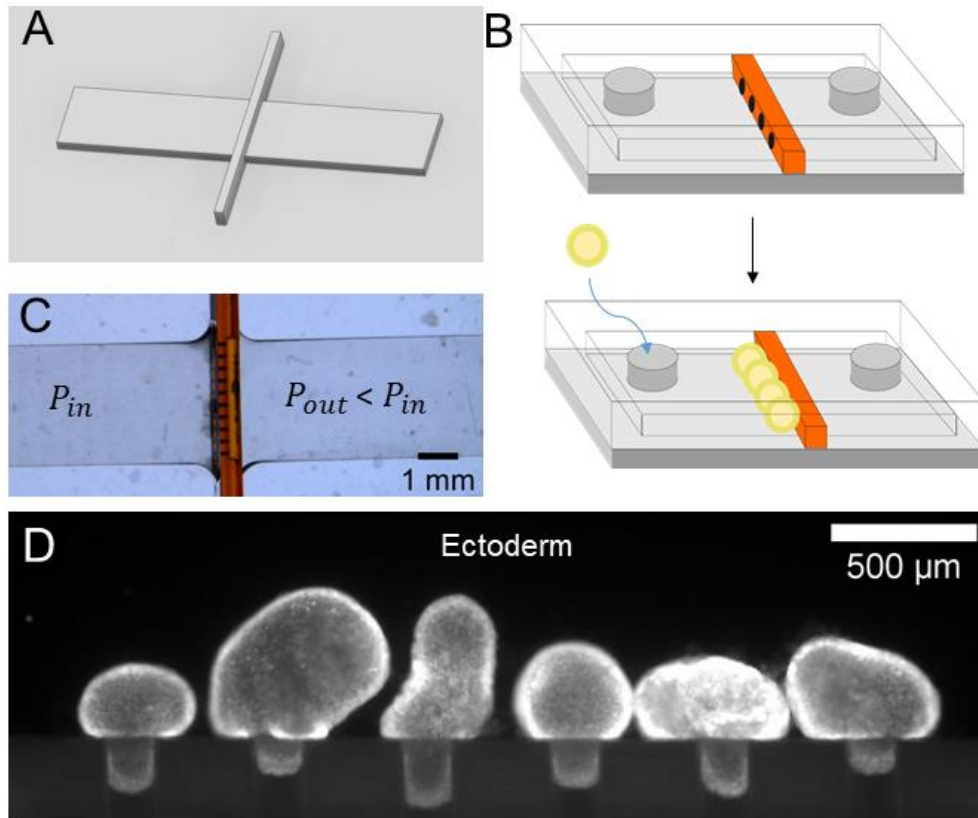
351 **Results**

352 *Multiplexed micro-aspiration setup*

353 In order to define and measure the rheological properties of *Hydra* tissue spheres, we settled on the
354 use of micro-aspiration experiments. These experiments are now well-established, robust and
355 measure rheological properties at length scales and frequencies relevant for the regenerative process.
356 In contrast with Atomic Force Microscopy (AFM), previously used to locally probe the stiffness of adult
357 *Hydras* (39), our experiments were particularly designed to study the mechanics of tissue spheres
358 during the osmotic oscillations which is why we employed elongations rather than indentations and
359 probed the large deformation regime. Their main drawback is their intrinsically low throughput. In
360 their original form, micro-aspiration experiments can only probe one sample at a time with a single
361 experiment running for around 1h (4, 33). We thus started by developing and adapting a microfluidics
362 device making use of removable inserts (36) to parallelize the experiment, in the same spirit as the
363 technique developed in (40) but with circular pipettes which prevent singularities and leakage. This
364 device was composed of two different objects. First, a microfluidic channel was designed to host the
365 samples, it was 3cm in length and 500 μ m in height (Fig 1A, Fig S1). Halfway through this channel and
366 perpendicular to it sat another channel meant to host the removable insert. This channel was 500 μ m
367 wide and 1mm high. This whole construct was then manufactured in polydimethylsiloxane (PDMS).

338 Most of the experiments presented here were carried out with removable inserts, microfabricated in
 339 dry film, bearing either 6 or 10 circular openings, 500 μ m or 300 μ m away from one another, 300 μ m in
 340 length and 100 μ m in diameter. Of note, these holes sat, vertically, at the midpoint of the samples'
 341 channel (250 μ m, Fig 1B). Tissue spheres aspirated in these holes therefore didn't touch either the
 342 bottom or the top of the PDMS channel which had no influence on the aspiration.

343



344

345 *Fig 1. Parallelized micro-aspiration experiments. A: Schematics of the mold for the PDMS channel (details on dimensions can*
 346 *be found in Fig S1). B: Representation of the principle of the experiment, the insert, in orange, effectively creates an array of*
 347 *cylindrical tunnels, equivalent to model micropipets. The tissue spheres, in yellow, flow towards these openings and, once*
 348 *sealed, get aspirated within. C: Snapshot of a mounted channel with an insert containing ten tunnels in parallel. D:*
 349 *Fluorescent imaging of the ectoderm of six samples aspirated within the tunnels.*

350 Using hydrostatic pressure, we applied a difference in pressure between the inlet and outlet of the
 351 main chamber (with $P_{in} > P_{out}$, see Fig S2) which initially created a flow of water from the Petri dish
 352 to the outlet of the chamber. Previously prepared *Hydra* tissue spheres were then manually pipetted
 353 into the entrance of the main channel. Thanks to the flow of water, the samples naturally aligned with
 354 the openings in the insert. Once all openings were blocked by a sample, the flow of water stopped and
 355 aspiration of the tissue spheres was observed (Fig 1D, Movie S1). Of note, in most experiments, the
 356 pressure difference was set to 0 as soon as the samples aligned with the holes so that a controlled step
 357 in pressure could be applied thereafter.

358 *Rheological behavior depends on applied stress.*

359 Using this multiplexed setup, we started by studying the response of newly formed *Hydra* tissue
 360 spheres to micro-aspirations in the kPa range using circular openings with a radius $R_p = 50\mu$ m. We

361 observed two different rheological responses depending on the applied pressure. Up to around 2.5kPa,
362 tissue spheres exhibited an elastic response in the sense that for a given applied pressure difference,
363 a tongue of tissue was aspirated within the holes which length increased in the first tens of seconds
364 but then saturated and remained constant over much longer time scales. When the pressure was
365 released, we also observed that the samples retrieved their original shape, and that aspiration and
366 release dynamics were very similar (Fig S3), consistent with the reversible behavior of an elastic
367 material. To further make sure that we were not missing a viscous behavior at longer time scales, we
368 managed to trap tissue spheres for tens of hours in this elastic phase without any indication of tissue
369 flow (Movie S2).

370 However, when we increased the applied pressure, we started observing samples flowing inside the
371 holes, a signature of a viscous behavior. This demonstrates a real change in mechanical behavior of the
372 tissue spheres akin to the behavior of yield stress fluids and requiring two different experimental and
373 theoretical approaches.

374 Finally, at even higher applied stresses, we started observing rupture of the tissue and cell
375 detachments, reminiscent of the rupture observed under normal phase I osmotic oscillations (Movie
376 S3). Since all three behaviors seemed relevant to understand the mechanical behavior of normally
377 regenerating tissue spheres, we decided to characterize them all, as well as the pressure thresholds
378 between these different phases.

379

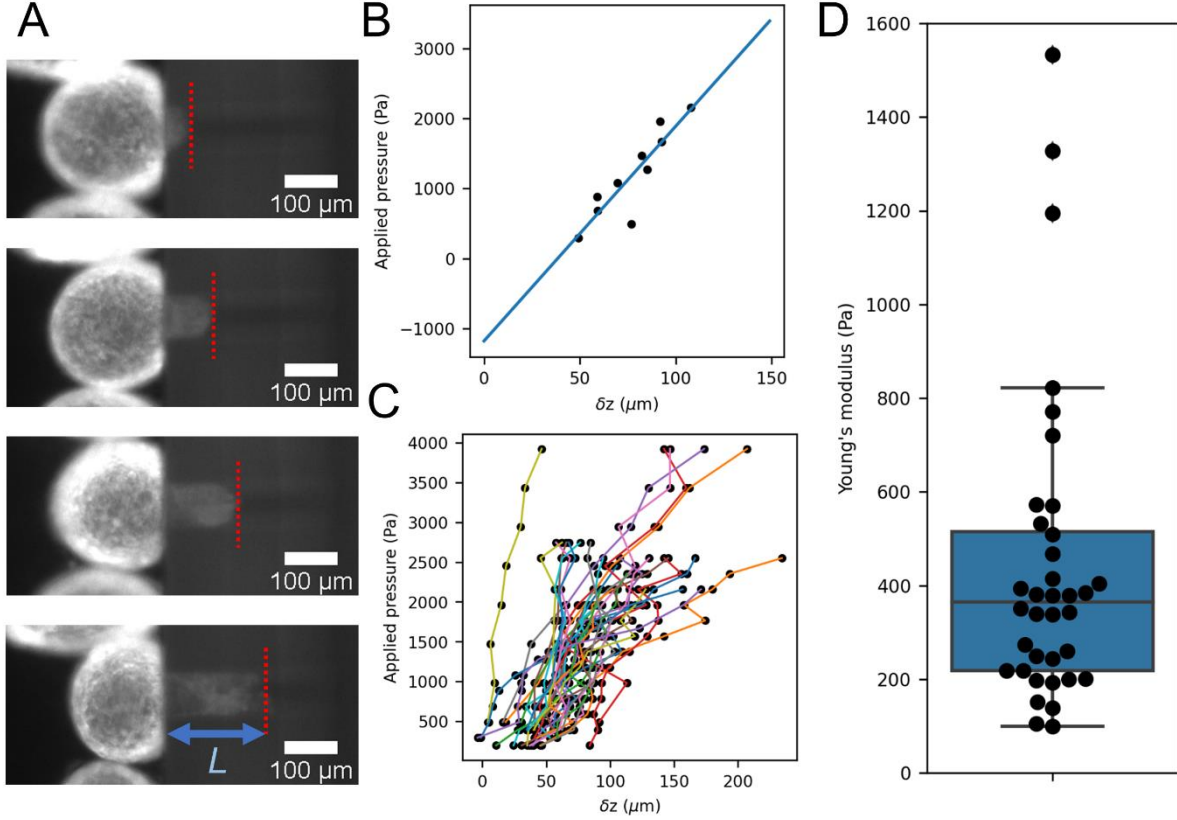
380 *Elastic behavior at low applied stresses.*

381 To characterize the elastic behavior of the tissue spheres, we applied steps of pressure on the same
382 samples and recorded, for each step and for each sample in an experiment, the length of the aspirated
383 tongue L (Fig 2A). We monitored the samples closely to make sure they did not start flowing in the
384 holes, in which case their analysis was stopped. As expected, we found that the higher the pressure,
385 the longer the aspirated tongue (Fig 2A). To estimate a Young's modulus from these measurements,
386 we needed a rheological model of an elastic spherical shell of mean radius R_0 and thickness h_0 .

387 As is the case for cell aggregates, we expect that intercellular adhesion and cellular cortical tension will
388 contribute to an effective surface tension of the *Hydra* tissue spheres, which we denote by γ .

389 A pressure difference ΔP through the micropipette of radius R_p generates an aspiration force $f_p =$
390 $\pi R_p^2 \Delta P$. For an aspirated length L , the free energy F of the aspirated shell reads:

$$F = 2\gamma S - f_p L \quad (1)$$



391 *Fig 2. Elastic behavior quantification. A: snapshots of one tissue sphere aspirated in a hole at increasing pressure steps (top*
 392 *to bottom). The dashed red line shows the position of the aspirated tongue at each step and L is the aspirated length. B:*
 393 *Linear relationship between the applied pressure and δz for a single tissue sphere and 10 different pressure steps showing*
 394 *both the linear behavior (fit as a blue line) and negative intercept. C: Same measurement as in B, repeated on $n=36$ tissue*
 395 *spheres, each represented by a differently colored line. D: distribution of Young's moduli obtained by this method. Black dots*
 396 *are individual measurements, the box plot shows the median and quartiles of the distribution. We found $E = (4.4 \pm 3.3) \cdot 10^2$*
 397 *Pa (mean \pm standard deviation, $n=36$).*

398 Where S denotes the surface area of the sample and the factor 2 takes into account the presence of
 399 two interfaces. Assuming incompressibility, the volume V of the sample is constant. For small enough
 400 deformations during aspiration, we expected that the thickness h_0 of the sample would remain
 401 constant. To validate this hypothesis, we performed spinning disk confocal microscopy on normal
 402 tissue spheres and aspirated ones to reconstruct their 3d structure and estimate their thicknesses. We
 403 found $h_0 = 20 \pm 5 \mu m$ in both cases and didn't observe any clear change due to micro aspiration (Fig
 404 S4). Since $V = Sh_0$, the surface area of the sample also remains constant $dS = 0$. In other words, in
 405 the case of the small deformations of an incompressible shell of approximately constant thickness,
 406 tissue surface tension does not contribute to the total force f exerted on the sample:

$$f = -\frac{dF}{dL} = -2\gamma \frac{dS}{dL} + f_p = f_p = \pi R_p^2 \Delta P \quad (2)$$

407 Indeed, our experiments do not exhibit a clear half-sphere of radius R_p , typical of the early entry of a
 408 full cell aggregate into a micropipette (41). In addition, (33) elegantly demonstrates that surface
 409 tension manifests itself as a critical pressure below which no aspiration is observed and which needs
 410 to correct all subsequent applied pressures. The fact that we observed aspirations with applied
 411 pressures as low as 200Pa also indicates that surface tension can largely be ignored in our experiments.

412 The rest state of the system is simply that of the initial spherical shell of radius R_0 and due to that
 413 initial curvature, the displacement of the tip of the tissue tongue inside the micropipette δz reads:

$$\delta z = L - (R_0 - \sqrt{R_0^2 - R_p^2}) \quad (3)$$

414 As expected for an elastic material, we found a linear relationship between this displacement and the
 415 applied pressure ΔP (Fig 2B). We also noticed that for many samples, our linear fits had negative
 416 intercepts at the origin. This could be the signature of a stress-stiffening effect by which the effective
 417 Young's modulus would increase with the applied pressure, *i.e.* the existence of non-linear elastic
 418 behavior, as observed in suspended cell monolayers (42).

419 To take into account this observation, we turned to a nonlinear, hyperelastic description of the *Hydra*
 420 tissue, using, as previously advocated by (43), the Saint-Venant Kirchhoff model, defined by the strain
 421 energy function:

422

$$W(E) = \frac{\lambda}{2} \text{Tr}(\bar{\bar{E}})^2 + \mu \text{Tr}(\bar{\bar{E}}^2) \quad (4)$$

423 where λ and μ denote the Lamé coefficients, and $\bar{\bar{E}}$ is the Green-Lagrange strain tensor (37). This
 424 equation reduces to its linear elastic counterpart for small deformations, when $\bar{\bar{E}} \approx \bar{\bar{\epsilon}}$ with $\bar{\bar{\epsilon}}$ the
 425 infinitesimal strain tensor. Of note, the Saint-Venant Kirchhoff model is defined by the same
 426 parameters as would be the case for a linear elastic material, with the following classical relationships
 427 between Lamé coefficients and other elastic moduli: E and ν , respectively, the Young's modulus and
 428 the Poisson's ratio of the sample.

429

$$E = \mu \frac{3\lambda + 2\mu}{\lambda + \mu} \quad (5)$$

$$\nu = \frac{\lambda}{2(\lambda + \mu)} \quad (6)$$

430 In analogy with the behavior of linearly elastic thin spherical shells (see for instance (44)), and taking
 431 into account the non-zero intercept ΔP_0 of the linear fits of our data (Fig 2C, $\Delta P_0 =$
 432 $(-6.4 \pm 1.3) \cdot 10^2 \text{ Pa}$), we expect that the displacement and the applied pressure should be related
 433 according to:

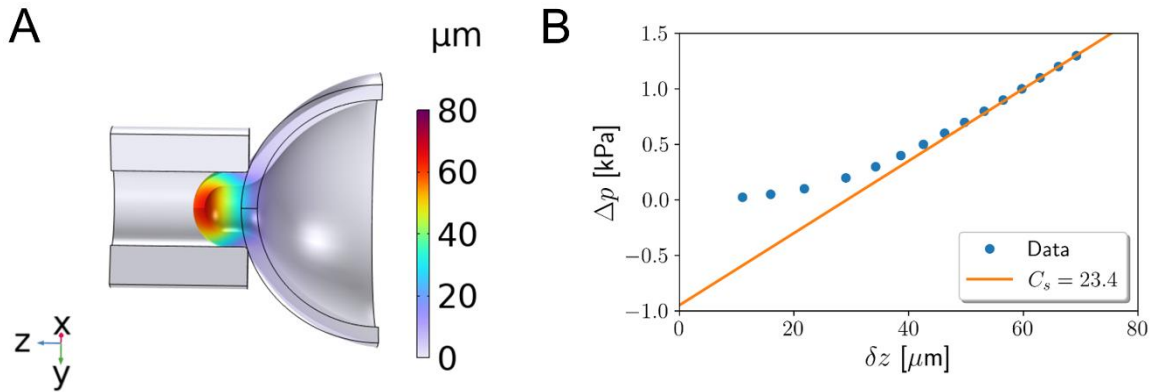
$$\Delta P = C_s \frac{2Eh_0}{(1 - \nu)R_0^2} \delta z + \Delta P_0 \quad (7)$$

434

435 Where C_s is a dimensionless, geometry-dependent pre-factor. Of note, in this description, we ignore
 436 the complex composite structure of the shell which includes two different epithelial layers as well as
 437 the extra-cellular matrix.

438 In order to estimate C_s , we performed numerical simulations of micro-aspiration of hyperelastic
 439 spherical shells by a single cylindrical tunnel (Fig 3A). In our experimental data, we had $R_0 = 162 \pm$
 440 $28 \mu\text{m}$ (mean \pm standard deviation, $n=36$) and $h_0 = 20 \pm 5 \mu\text{m}$. As we had no measurement of the
 441 Poisson's ratio of our samples, we made an assumption of near incompressibility and set $\nu = 0.495$.
 442 Based on these values, we submitted spherical shells to applied pressure ΔP and measured the

443 resulting δz . As shown in Fig 3B, these simulations confirmed the linear relationship between ΔP and
 444 δz over a similar range of applied pressures and displacements and allowed to estimate C_s thanks to
 445 the knowledge of the value of E and ΔP_0 . Of note, other element-based numerical simulations were
 446 performed with parameters varying in the following ranges: $25 \leq \Delta P \leq 2000 Pa$, $15 \leq h_0 \leq 25 \mu m$
 447 , $130 \leq R_0 \leq 200 \mu m$, $200 \leq E \leq 750 Pa$ and with a fillet size varied between 2 and $10 \mu m$. Overall,
 448 our estimate of the coefficient C_s reads $C_s = 23.4 \pm 4.3$, in the vicinity of 23, suggesting that the order
 449 of magnitude of our results was unaffected by uncertainties on these different parameters. Further,
 450 our estimate of the intercept in these simulations is $\Delta P_0 = (-8.5 \pm 2.1) \cdot 10^2 Pa$, consistent with the
 451 above experimental values.



452

453 *Fig 3. Numerical simulations. A: Graphical representation of the deformed shell under. The color code represents the z-*
 454 *component of the displacement field. Black lines delineate the reference state of the system. Parameter values for these*
 455 *simulations are $R_0 = 160 \mu m$, $h_0 = 20 \mu m$, $E = 440 Pa$, $\nu = 0.495$, $\Delta P = 10^3 Pa$. B: applied pressure Δp versus the*
 456 *displacement δz for the same parameter values. The slope of the fitted line, in orange, corresponds to a value $C_s = 23.4$.*

457 Thanks to our multiplexed setup, we were able to measure up to six samples in parallel in one sitting.
 458 By then repeating these experiments eight different times, we achieved a total of 36 tissue spheres
 459 (Fig 2C) characterized in that phase. For each of them, we extracted the slope of the linear relationship
 460 between ΔP and δz , its initial radius R_0 and finally obtained a quantitative measurement of its Young's
 461 modulus using Eq 7 (Fig 2D). From the above estimate of the coefficient C_s , we found the Young's
 462 modulus of *Hydra* tissue spheres to be $(4.4 \pm 3.3) \cdot 10^2 Pa$ (mean \pm standard deviation, $n=36$).

463 We also observed a large variability in our measurements of individual Young's moduli (Fig 2D) which
 464 could hint at subtle effects missed by our averaging approach. The different tissue spheres could differ
 465 in initial size or degree of inflation since we do not precisely control the time they need to fold back
 466 into tissue spheres after being cut. To better understand this variability, we looked at the correlation
 467 between the samples' radii and their extracted elastic moduli. We indeed found a positive correlation,
 468 albeit weak, between these measurements (Fig S5). We then asked whether this correlation could be
 469 due to differences in inflation state. To do so, we ran new experiments measuring the elastic moduli
 470 of tissue spheres whose oscillations were blocked by the addition of 70mM sucrose in the outside
 471 medium. Doing so, we found 1- that the obtained elastic moduli distributions in both cases were not
 472 statistically different (Fig S6) and 2- that addition of sucrose abolished neither the weak positive
 473 correlation between size and elasticity (Fig S5) nor the existence of stiffer samples. We conclude that
 474 some, but not all, of the observed variability comes from differences in initial size rather than inflation
 475 rate between different samples. A better understanding of this dependency would be a natural
 476 continuation of this work.

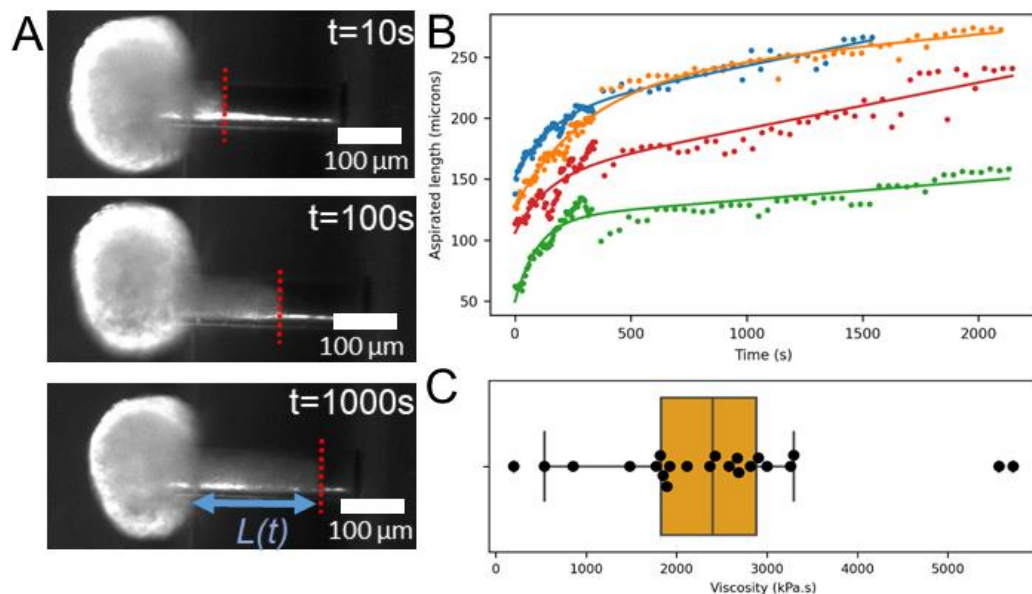
477 In addition, we validated the relevance and precision of our experimental procedure by repeating
 478 elastic measurements under two chemical modulations: EDTA and blebbistatin. EDTA is a chemical
 479 well-known for weakening cell-cell adhesions in many epithelial tissues (45) whereas blebbistatin

480 inhibits acto-myosin contractility (46). We expected that both treatments should lower the samples'
 481 elasticity either by allowing larger tissue-scale deformations in the case of EDTA or cell-scale
 482 deformations in the case of blebbistatin by making the actin cortex of each cell softer. We indeed found
 483 a clear and statistically significant decrease in Young's modulus with both treatments (Fig S6, EDTA:
 484 $E=(3.0 \pm 2.0) \cdot 10^2$ Pa, blebbistatin: $E=(1.9 \pm 0.7) \cdot 10^2$ Pa), confirming that the observed elastic behavior
 485 of the tissue spheres stems from a complex interplay of single cell mechanics and tissue structure.

486 Overall, we characterized in a quantitative way, the response of *Hydra* tissue spheres to applied
 487 stresses and found them to behave hyperelastically, *i.e.* as soft ($E \sim 500$ Pa) elastic materials at small
 488 deformations, in line with AFM measurements on adult *Hydras* (39), while they become increasingly
 489 stiffer as the applied pressure increases.

490 *Visco-elastic behavior at intermediate applied stresses*

491 We then turned our attention to the next observed behavior which involved flowing of the samples
 492 within the tunnels. This visco-elastic behavior has long been observed and studied, both on single cells
 493 (32, 47, 48) and multi-cellular spheroids (4, 33, 41). We followed the usual approach in that situation
 494 where a single, controlled pressure step was applied in the device at $t=0$ after the samples were already
 495 placed close to the entrance of the tunnels. The dynamics of the aspirated length $L(t)$ was then
 496 recorded by fluorescent microscopy for tens of minutes (Fig 4A). It was important to keep recording
 497 on these longer time scales in order to be certain of the behavior adopted by each sample as a very
 498 slow flow could easily be misinterpreted as an elastic response if the observation time scale was too
 499 short.



500
 501 *Fig 4. Visco-elastic flow at large applied pressure. A: snapshots of a tissue sphere showing flow at a constant applied*
 502 *pressure. B: four examples of typical dynamics of the flow as a function of time. Dots of different colours represent four*
 503 *independent samples and solid lines fits by Eq 8. C: distribution of measured viscosities as black dots with box-whisker plot in*
 504 *orange. We found it to be $(2.4 \pm 1.2) \cdot 10^6$ Pa.s (mean \pm standard deviation, $n=22$).*

505 Since the behavior is qualitatively similar to that observed for cellular aggregates aspirated in
 506 cylindrical micropipets, we first applied the well-known modified Maxwell model developed in (33).
 507 Briefly, a dashpot representing tissue scale viscosity is combined in series with a standard linear solid
 508 unit incorporating cell scale viscoelasticity as well as tissue scale elasticity. We fitted our data with the
 509 form of $L(t)$ predicted by this model:

$$L(t) = \delta \left(1 - \kappa e^{-\frac{t}{\tau}} \right) + Ut \quad (8)$$

510

511 Where δ is linked to the short-term elastic deformation, κ is a constant linked to the relative
 512 contributions of the two springs in the standard linear solid unit, τ is the cell scale visco-elastic
 513 relaxation-time and U is the long term viscous flow rate. We found that this functional form was also
 514 relevant to describe our data quantitatively (Fig 4B) despite the morphological differences between
 515 our samples (empty shells) and ball-shaped aggregates (full spheres). We could however employ a
 516 scaling argument similar to the one applied in this model to estimate an order of magnitude of the
 517 effective viscosity of our samples. As for cellular aggregates, friction between the tissue and the
 518 tunnels walls can be neglected since the long-term aspirated length scales not as the square-root of
 519 time, but as a linear function of time. Assuming that dissipation is due to cell rearrangements at the
 520 entrance of the tunnels thus acting on a length scale of order R_p , the total dissipative force can
 521 therefore be approximated as $f_{viscous} \approx C_v R_p U \eta$ with η the viscosity of the samples and C_v a
 522 dimensionless, geometrical pre-factor equal to 3π for a ball-shaped aggregate and unknown in our
 523 geometry. This viscous force has to be balanced by the aspiration force which we have shown to be
 524 $f_p = \pi R_p^2 \Delta P$ which leads to:

$$\eta \approx \frac{\Delta P R_p}{U C_v} \quad (9)$$

525 To the best of our knowledge the computation of C_v has never been done for spherical viscous shells
 526 and in the same geometry as the one in our experiments. We will see below that a precise
 527 measurement of this effective viscosity has little impact on the description of *Hydra* osmotic
 528 oscillations and we limited ourselves to obtaining an order of magnitude estimation of the viscosity
 529 by taking $C_v = 1$. Note that assuming that tissue scale dissipation acts across the thickness h of this
 530 multilayered tissue does not change our estimate as h and R_p have the same order of magnitude.
 531 Again, our microaspiration setup allowed us to efficiently gather data on multiple samples and
 532 combining them, we found an estimation of the viscosity of $(2.4 \pm 1.2) \cdot 10^6$ Pa.s (mean \pm standard
 533 deviation, $n=22$, Fig 4C).

534 The full dynamics of aspiration in that phase can also yield measurements of the Young's modulus of
 535 the samples. In line with our analysis of the purely elastic response, we expect here that:

$$\Delta P = C_s \frac{2Eh}{(1-\nu)R_0^2} \delta(1-\kappa) \quad (10)$$

536

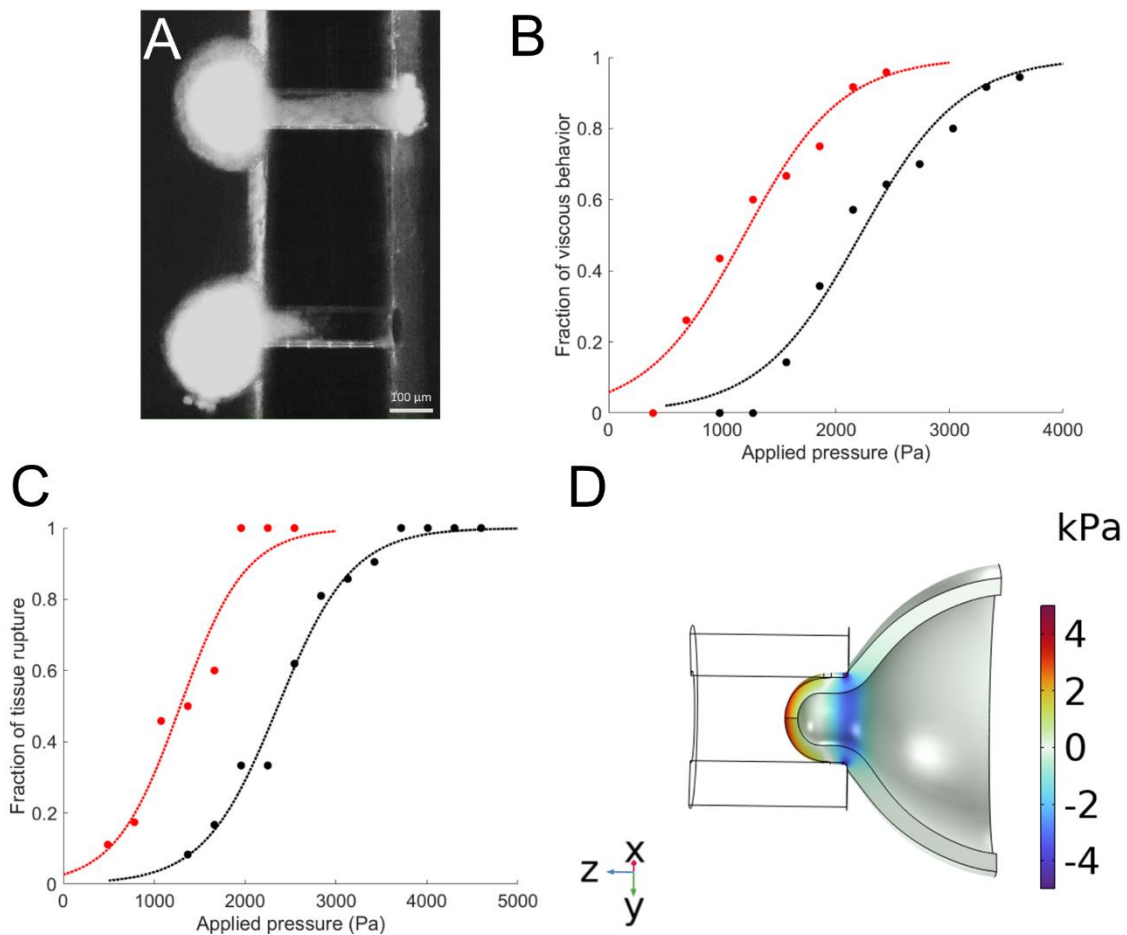
537 Similar to Eq. 7 with $\delta(1-\kappa)$ the immediate elastic aspirated length in the notation of this model.
 538 Applying this relationship to the results of our fitted data, we found $E = (3.7 \pm 1.6) \cdot 10^2$ Pa (Fig S7),
 539 in accordance with our results on the purely elastic behavior of *Hydra* tissue spheres.

540 These visco-elastic experiments were made difficult by the fact that in many instances, we observed,
 541 in addition to the flow of the samples, cells detaching at the tip of the aspirated tongue, indicative that
 542 the tissues were already rupturing. These cases were obviously discarded from the analysis, limiting
 543 the amount of available data. Overall, it appeared that at these applied pressures, the visco-elastic
 544 phase and rupture were often occurring together.

545

546 *Critical pressures between different behaviors*

547 To further study this question, we then turned our attention to the critical pressures required to
 548 transition between the three regimes: elastic, viscoelastic and rupture. We often found cases where,
 549 for a given applied pressure, a fraction of the samples loaded in the same setup would behave as elastic
 550 solids while other behaved as viscoelastic fluids (Fig 5A). To characterize the first critical pressure, we
 551 therefore measured, at different applied pressures, the fraction of samples showing a viscoelastic
 552 versus elastic behavior while discarding rupturing ones. We fitted this fraction as a function of the
 553 applied pressure by a sigmoid (see Materials and Methods, Fig 5B). We defined the critical pressure as
 554 the center of this sigmoid where 50% of samples had switched from one behavior to the other.
 555 Following this method, we found the critical pressure between elastic and viscoelastic behaviors to be
 556 2.22 ± 0.14 kPa (error bars represent the 95% confidence interval of the sigmoid fit). We then employed
 557 the same strategy to characterize the critical pressure leading to tissue rupture (Fig 5C). We found it
 558 to be 2.37 ± 0.08 kPa, very close to the previous one confirming our intuition that both behaviors were
 559 linked together and that tissue flows inside the holes were already a signature of their lack of integrity.



560
 561 *Fig 5. Critical stresses between different mechanical behaviors. A: snapshot of an experiment showing two similar samples*
 562 *displaying different behaviors at the same applied pressure. B: Quantification of the fraction of viscous behavior at different*
 563 *applied pressures. C: quantification of the fraction of samples showing tissue rupture as a function of applied pressure. In B*
 564 *and C, dots are data and solid lines fit by a sigmoid function. In black are control samples and in red samples pretreated with*
 565 *2mM EDTA. Each point is derived from 18 to 40 samples stemming from 8 different experiments in B and 4 in C. D:*
 566 *Numerical simulations. The color code represents the hoop stress within the aspirated shell when aspirated at a pressure of*
 567 *1kPa. Parameter values are the same as in Fig. 3.*

568 The most natural explanation for this lack of integrity is the weakening and rupture of cell-cell contacts
 569 since we often observed detachment of single cells, making a possible deadhesion of the tissues to the

570 extracellular matrix unlikely. To verify this hypothesis, we repeated our measurement of the critical
 571 pressures required to induce either a viscous behavior or tissue rupture on tissue spheres pre-treated
 572 with 2mM EDTA as we expected this treatment to facilitate the induction of a viscous behavior and
 573 tissue rupture. This is indeed what we found with a shift of the critical pressure from 2.22 kPa to 1.19
 574 ± 0.16 kPa for the viscous behavior (Fig 5B) and from 2.37 kPa to 1.29 ± 0.19 kPa for rupture (Fig 5C).

575 It is important to note that all these values are difference of pressure applied between the two sides
 576 of the holes and not necessarily the stress actually acting in the tissue called the hoop stress. Said
 577 differently, the applied pressure required to rupture the tissue shouldn't be directly taken as a
 578 measurement of the ultimate tensile stress of the whole tissue. To relate the applied pressure and
 579 internal tissue stresses, we turned again to numerical simulations. Within the range of parameters
 580 studied, the maximal values of the hoop stress we found was on the order of a 2-7 kPa (see Fig 5D for
 581 an example).

582 Since in normal conditions, the threshold for viscous behavior cannot be clearly distinguished from the
 583 threshold for rupture, our measurements revealed that up to rupture, which is also observed in normal
 584 osmotic oscillations, regenerating *Hydra* tissue spheres behave as hyperelastic spherical shells with a
 585 stress stiffening effect.

586

587 *Mechanical description of oscillating Hydra tissue spheres during regeneration.*

588 After folding back into a spherical shape, regenerating *Hydra* tissue spheres experience a series of
 589 swelling-rupture cycles which have been shown to be driven by the difference in osmolarity between
 590 the inside and outside of the spheres. These oscillations are required for the proper morphogenesis
 591 and regeneration of the samples although their exact role remains unknown. Thanks to previous work
 592 on the topic (5, 49) and our own mechanical characterization, we can provide a good understanding of
 593 the internal tissue mechanics occurring during this phase.

594 The key quantity is the circumferential or hoop stress, noted here σ , which is known to control tissue
 595 integrity and to be able to alter key biological processes. It is given for an incompressible, thin elastic
 596 spherical shell of a Saint-Venant Kirchhoff material (see (37, 38) and the Methods section) by the
 597 expression:

$$\sigma = \left(\alpha^4 - \frac{1}{\alpha^2} \right) \left(\left(\frac{\lambda}{2} + \mu \right) \left(2 + \frac{1}{\alpha^6} \right) - \left(\frac{3\lambda}{2} + \mu \right) \frac{1}{\alpha^2} - \mu \right) \quad (11)$$

598

599 With $\alpha = \frac{R(t)}{R_0}$ is the stretch ratio, and λ and μ the Lamé coefficients. This gives the nonlinear
 600 constitutive relationship between stresses and strains in the spherical shell during the oscillations
 601 which allows to directly extract the hoop stress from a measurement of the radius of the sample as a
 602 function of time (see Fig S8). One can check that Eq 11 reduces to the relationship expected of a linear
 603 elastic thin shell in the limit of small deformations where $|R - R_0| \ll R_0$:

$$\sigma = \frac{E}{1 - \nu} \cdot \frac{R - R_0}{R_0} \quad (12)$$

604 At larger deformations, the tissues get stiffer and the experimentally measured values of E imply that,
 605 in this regime, the differential elastic modulus $\frac{\partial \sigma}{\partial \alpha}$ is on the order of 10kPa, in accordance with previous
 606 estimates of the sphere's stiffness during osmotic oscillations (5).

607 Based on our experimental observations, we will approximate our samples as hyperelastic, symmetric,
608 spherical shells of time-dependent thickness, $h(t)$ and radius $R(t)$ (Fig S8) submitted to a difference
609 in osmolite concentrations ($C_{in} - C_{out}$) > 0 . The fact that the spheres are constantly swelling
610 indicates a constant influx of water into their lumen and that the system never reaches osmotic
611 equilibrium. Furthermore, Kücken et al. (5) showed that the increase of $R(t)$ can be described by a
612 Darcy-type law of the form:

$$\frac{dV}{dt} = 4\pi R(t)^2 L_w k_b T (C_{in} - C_{out}) \quad (13)$$

613 Where $V(t)$ is the volume of the sphere, L_w is the water permeability coefficient, k_b is Boltzmann's
614 constant. The dynamics of swelling is thus entirely controlled by the influx of water which imposes a
615 constantly increasing deformation on the tissue sphere with a growth rate of the radius on the order
616 of $5\mu m/h$. This influx of water effectively inflates the sphere and creates a restoring elastic pressure
617 P_{el} which our mechanical description can also predict. Following the mechanics of spherical thin shells,
618 this pressure can be written as:

$$P_{el}(t) = \frac{2h(t)}{R(t)} \sigma(t) \quad (14)$$

619 Using the experimental measurement of E we previously obtained and the same value of the Poisson's
620 ratio, $\nu = 0.495$, we were able to approximate the values of λ and μ ($\mu = 148Pa, \lambda = 100\mu$). Using
621 these values and experimental measurements of $\alpha(t)$ during the oscillations allowed us to estimate
622 $\sigma(t)$ and we found it to be on the order of a few kPa (Fig S8). Most notably, this approach allowed us
623 to estimate the critical stress, within the tissue, to induce rupture and we found it to be on the order
624 of 4-6kPa. This is the same order of magnitude as the maximum hoop stress obtained by numerical
625 simulations of the elastic deformation during micropipette aspiration. Overall, we demonstrate that
626 our results allow to map measurements of the radius of the tissue spheres, which are experimentally
627 easy to access, into both internal stresses within the tissue and restoring elastic pressure acting on the
628 fluid inside of the spheres.

629

630 Discussion

631 The effect of stretching on the biology of epithelial tissues has emerged in recent years as an important
632 topic in developmental biology since developing organisms often undergo substantial deformations
633 which have an increasingly well recognized impact on their biological response. In the context of *Hydra*
634 regeneration, Ferenc et al. observed that the levels of expression of Wnt ligands, especially HyWnt3,
635 are directly linked to the cumulative amount of stretching in the tissue (20). In a similar fashion,
636 stretching adhering monolayers of epithelial cells has been shown to induce cell division through the
637 activation of Piezo1 mechanosensitive ion channels (22) to restore proper cell density. Anisotropic
638 deformations also have a large impact on the tissue as uniaxial cyclic stretch leads to re-orientation of
639 the cells perpendicular to the direction of application (50, 51), as was observed for the mitotic spindle
640 of single cells (52). In many of these experiments, however, stresses within the tissues couldn't be
641 recorded after stretching.

642 One notable exception was achieved on freely suspended epithelia axially stretched with a measurable
643 force (42). This experimental setup allowed the authors to characterize the rheology of Madine–Darby

644 Canine Kidney (MDCK) monolayers. Interestingly, they observed a non-linear response with three
645 distinct phases: a linear one at small deformations with a small equivalent elastic modulus, a second
646 linear one for intermediate values of stresses and strains with much higher stiffness and a third one
647 corresponding to the rupture of the tissue. This behavior is very similar to the one we observed in this
648 work. The authors quantified the second phase by linear fitting of the stress-strain curves with negative
649 intercepts, as we did. Since they approximated the tissue in this phase as linearly elastic, they found a
650 high elastic modulus on the order of 20kPa, similar to our observations of *Hydra* tissue spheres
651 differential moduli at intermediate stresses. Finally, the authors found that the monolayer was able to
652 withstand deformations up to 60% before rupturing, a value slightly higher than what we observed for
653 *Hydra* tissue spheres. Our results are thus well in line with these observations, and both ask the
654 question of the biological origin of the non-linear behavior of epithelial tissues which remains elusive
655 for the moment.

656 Regarding the specific rheology of *Hydra*, our work is the first to focus specifically on regenerating
657 tissue spheres but measurements exist on full adults. In (39), the authors employed a modified AFM
658 experimental procedure to generate indentations on fixed, full *Hydras* with a 20 μ m wide sphere. By
659 co-recording the deformation of the tissue and the force exerted, they observed a linear relationship
660 between both which allowed them to estimate the elastic modulus of these samples. One key result
661 was the observation that the elasticity of the body column was inhomogeneous, the tissue being
662 roughly three times stiffer near the head than close to the foot. Although it is a well-known fact that
663 different experimental approaches can lead to different estimations of rheological properties (53) and
664 potential differences between fully mature *Hydras* and tissue spheres, our results are in good
665 agreement with this work. Indeed, in the softest part of the adult, which we would imagine to most
666 resemble tissue spheres, the authors report an elastic modulus on the order of 400Pa. Since the
667 deformations involved are of a few microns, we expect these measurements to probe the small
668 deformation regime where our own measurements also find an elastic modulus on the order of 400Pa.
669 In the large deformation regime, our results also confirm those of (5) which, in order to properly
670 reproduce the osmotic oscillations based on chemical considerations implemented an elastic modulus
671 $E = 11kPa$. Still, AFM measurements on tissue spheres, albeit technically challenging, would be a
672 great complement to our work to obtain a comprehensive understanding of the mechanics of these
673 samples.

674 Another consequence of our focus on osmotic oscillations and tissue-scale mechanics is that our
675 mechanical description of the tissue spheres assumes a homogenous shell, ignoring the complex three-
676 layer (endoderm-extra-cellular matrix-ectoderm) structure of the samples. It is clearly possible that
677 the deformations and stresses induced by the osmotic oscillations are differently transmitted and have
678 different effects on these different layers, a refinement which will need to be addressed in the future.
679 In particular, it was recently suggested that the mechanical properties of the ECM, which were not
680 specifically measured here, could be linked to morphogenesis in *Hydra* (54).

681 Since we believe deformations and stresses within the tissues to be central in the morphogenesis of
682 *Hydra*, another natural question is the existence of inhomogeneities of deformations, forces or
683 rheological properties within the same sample. Mechanical fluctuations, if coupled to biochemistry,
684 could well be sufficient to destabilize a reaction-diffusion system into a patterned state. Our own
685 experimental setup could allow to measure fluctuations in rheological properties by repeating
686 measurements on the same samples with small holes but at different locations, the main limitation
687 being the lack of control of the position of the tested zone. In the same logic, it has been proposed
688 that the amount of HyWnt3 protein could locally affect the Young's modulus of the tissue (25) which
689 could couple the chemical patterning of the head organizer and morphogenesis. If the future location

690 of the head becomes softer, the homogenous osmotic pressure will deform this region more, leading
691 to a deviation from a spherical shape to an oblong one. Here too, our setup could allow to measure
692 the Young's modulus of tissue spheres overexpressing HyWnt3 to directly test this hypothesis.

693

694 **Conclusion**

695 In this work, we have employed an original parallelized microaspiration device to characterize the
696 mechanical behavior of *Hydra* tissue spheres. Although we found different rheological behaviors
697 depending on the applied stresses, the main result we obtained is that during normal osmotic
698 oscillations, these samples behave largely as hyperelastic thin shells, displaying a nonlinear, stress-
699 stiffening rheology. Doing so, we also obtained quantitative measurements of their relevant
700 rheological parameters ($E = (4.4 \pm 3.3) \cdot 10^2$ Pa, $\eta = (2.4 \pm 1.2) \cdot 10^6$ Pa.s, critical applied
701 pressures for viscous behavior and tissue rupture). This allowed us to estimate the stresses acting
702 within the tissues and, in particular, the critical stress at which they start rupturing. These results, by
703 allowing a quantification of both stresses and deformations during *Hydra* regeneration, will hopefully
704 pave the way for quantitative approaches aiming at correlating these mechanical cues with molecular
705 ones to understand *Hydra* patterning as an integrated mechano-biochemical process.

706

707 **Author contributions**

708 OCE designed the research. ZBM, MD and PJ manufactured the inserts. TP, ABB, PM and OCE
709 performed the research and analyzed data. TP, PM and OCE wrote the manuscript which was
710 reviewed by all authors.

711 **Declaration of interests**

712 The authors have declared no conflict of interest.

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717 **References**

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- 719 1. Galliot, B., and V. Schmid. 2002. Cnidarians as a model system for understanding evolution
720 and regeneration. *Int. J. Dev. Biol.* 46:39–48.
- 721 2. Technau, U., and R.E. Steele. 2011. Evolutionary crossroads in developmental biology:
722 Cnidaria. *Development.* 138:1447–58.
- 723 3. Gierer, A., S. Berking, H. Bode, C.N. David, K. Flick, G. Hansmann, H. Schaller, and E. Trenkner.
724 1972. Regeneration of hydra from reaggregated cells. *Nat. New Biol.* 239:98–101.
- 725 4. Cochet-Escartin, O., T.T. Locke, W.H. Shi, R.E. Steele, and E.-M.S. Collins. 2017. Physical
726 Mechanisms Driving Cell Sorting in Hydra. *Biophys. J.* 113:2827–2841.
- 727 5. Kücken, M., J. Soriano, P.A. Pullarkat, A. Ott, and E.M. Nicola. 2008. An osmoregulatory basis
728 for shape oscillations in regenerating hydra. *Biophys. J.* 95:978–85.

- 729 6. Fütterer, C., C. Colombo, F. Jülicher, and A. Ott. 2003. Morphogenetic oscillations during
730 symmetry breaking of regenerating *Hydra vulgaris* cells. *Europhys. Lett.* 64:137.
- 731 7. Broun, M., L. Gee, B. Reinhardt, and H.R. Bode. 2005. Formation of the head organizer in
732 hydra involves the canonical Wnt pathway. *Development.* 132.
- 733 8. Bode, H.R. 2009. Axial Patterning in Hydra. *Cold Spring Harb. Perspect. Biol.* 1.
- 734 9. Böttger, A., and M. Hassel. 2012. Hydra, a model system to trace the emergence of
735 boundaries in developing eumetazoans. *Int. J. Dev. Biol.* 56:583–91.
- 736 10. Soriano, J., C. Colombo, and A. Ott. 2006. Hydra molecular network reaches criticality at the
737 symmetry-breaking axis-defining moment. *Phys. Rev. Lett.* 97:258102.
- 738 11. Turing, A.M. 1952. The Chemical Basis of Morphogenesis. *Philos. Trans. R. Soc. B Biol. Sci.*
739 237:37–72.
- 740 12. Marcon, L., and J. Sharpe. 2012. Turing patterns in development: What about the horse part?
741 *Curr. Opin. Genet. Dev.* 22:578–584.
- 742 13. Schweisguth, F., and F. Corson. 2019. Self-Organization in Pattern Formation. *Dev. Cell.*
743 49:659–677.
- 744 14. Gierer, A., and H. Meinhardt. 1972. A theory of biological pattern formation. *Kybernetik.*
745 12:30–9.
- 746 15. Nakamura, Y., C.D. Tsiairis, S. Özbek, and T.W. Holstein. 2011. Autoregulatory and repressive
747 inputs localize Hydra Wnt3 to the head organizer. *Proc. Natl. Acad. Sci. U. S. A.* 108:9137–42.
- 748 16. Augustin, R., A. Franke, K. Khalturin, R. Kiko, S. Siebert, G. Hemmrich, and T.C.G. Bosch. 2006.
749 Dickkopf related genes are components of the positional value gradient in Hydra. *Dev. Biol.*
750 296:62–70.
- 751 17. Vogg, M.C., L. Beccari, L. Iglesias Ollé, C. Rampon, S. Vriz, C. Perruchoud, Y. Wenger, and B.
752 Galliot. 2019. An evolutionarily-conserved Wnt3/ β -catenin/Sp5 feedback loop restricts head
753 organizer activity in Hydra. *Nat. Commun.* 10:312.
- 754 18. Ziegler, B., I. Yiallourous, B. Trageser, S. Kumar, M. Mercker, S. Kling, M. Fath, U. Warnken, M.
755 Schnölzer, T.W. Holstein, M. Hartl, A. Marciniak-Czochra, J. Stetefeld, W. Stöcker, and S.
756 Özbek. 2021. The Wnt-specific astacin proteinase HAS-7 restricts head organizer formation in
757 Hydra. *BMC Biol.* 19:1–22.
- 758 19. Soriano, J., S. Rüdiger, P. Pullarkat, and A. Ott. 2009. Mechanogenetic coupling of Hydra
759 symmetry breaking and driven Turing instability model. *Biophys. J.* 96:1649–60.
- 760 20. Ferenc, J., P. Papasaikas, J. Ferralli, Y. Nakamura, S. Smallwood, and C.D. Tsiairis. 2021.
761 Mechanical oscillations orchestrate axial patterning through Wnt activation in Hydra. *Sci. Adv.*
762 7:6897.
- 763 21. Desprat, N., W. Supatto, P.-A. Pouille, E. Beaurepaire, and E. Farge. 2008. Tissue deformation
764 modulates twist expression to determine anterior midgut differentiation in *Drosophila*
765 embryos. *Dev. Cell.* 15:470–7.
- 766 22. Gudipaty, S.A., J. Lindblom, P.D. Loftus, M.J. Redd, K. Edes, C.F. Davey, V. Krishnegowda, and J.
767 Rosenblatt. 2017. Mechanical stretch triggers rapid epithelial cell division through Piezo1.
768 *Nature.* 543:118–121.
- 769 23. Pouille, P.-A., P. Ahmadi, A.-C. Brunet, and E. Farge. 2009. Mechanical signals trigger Myosin II
770 redistribution and mesoderm invagination in *Drosophila* embryos. *Sci. Signal.* 2:ra16.

- 771 24. Shinozuka, T., R. Takada, S. Yoshida, S. Yonemura, and S. Takada. 2019. Wnt produced by
772 stretched roof-plate cells is required for the promotion of cell proliferation around the central
773 canal of the spinal cord. *Dev.* 146.
- 774 25. Mercker, M., A. Köthe, and A. Marciniak-Czochra. 2015. Mechanochemical symmetry breaking
775 in Hydra aggregates. *Biophys. J.* 108:2396–407.
- 776 26. Brinkmann, F., M. Mercker, T. Richter, and A. Marciniak-Czochra. 2018. Post-Turing tissue
777 pattern formation: Advent of mechanochemistry. *PLoS Comput. Biol.* 14:e1006259.
- 778 27. Wang, R., T. Goel, K. Khazoyan, Z. Sabry, H.J. Quan, P.H. Diamond, and E.-M.S. Collins. 2019.
779 Mouth Function Determines the Shape Oscillation Pattern in Regenerating Hydra Tissue
780 Spheres. *Biophys. J.* 117:1145–1155.
- 781 28. Livshits, A., L. Shani-Zerbib, Y. Maroudas-Sacks, E. Braun, and K. Keren. 2017. Structural
782 Inheritance of the Actin Cytoskeletal Organization Determines the Body Axis in Regenerating
783 Hydra. *Cell Rep.* 18:1410–1421.
- 784 29. Wang, R., R.E. Steele, and E.M.S. Collins. 2020. Wnt signaling determines body axis polarity in
785 regenerating Hydra tissue fragments. *Dev. Biol.* 467:88–94.
- 786 30. Wang, R., A.L. Bialas, T. Goel, and E.M.S. Collins. 2023. Mechano-Chemical Coupling in Hydra
787 Regeneration and Patterning. *Integr. Comp. Biol.* 63:1422–1441.
- 788 31. Veschgini, M., F. Gebert, N. Khangai, H. Ito, R. Suzuki, T.W. Holstein, Y. Mae, T. Arai, and M.
789 Tanaka. 2016. Tracking mechanical and morphological dynamics of regenerating Hydra tissue
790 fragments using a two fingered micro-robotic hand. *Appl. Phys. Lett.* 108.
- 791 32. Evans, E.A. 1973. New Membrane Concept Applied to the Analysis of Fluid Shear- and
792 Micropipette-Deformed Red Blood Cells. *Biophys. J.* 13:941–954.
- 793 33. Guevorkian, K., M.-J. Colbert, M. Durth, S. Dufour, and F. Brochard-Wyart. 2010. Aspiration of
794 Biological Viscoelastic Drops. *Phys. Rev. Lett.* 104:1–4.
- 795 34. Hochmuth, R.M. 2000. Micropipette aspiration of living cells. *J. Biomech.* 33:15–22.
- 796 35. Lim, C.T., E.H. Zhou, and S.T. Quek. 2006. Mechanical models for living cells--a review. *J.*
797 *Biomech.* 39:195–216.
- 798 36. Landiech, S., M. Elias, P. Lapèze, H. Ajiyel, M. Plancke, A. Laborde, F. Mesnilgrente, D. Bourrier,
799 D. Berti, C. Montis, L. Mazonq, J. Baldo, C. Roux, M. Delarue, and P. Joseph. 2023. Parallel on-
800 chip micropipettes enabling quantitative multiplexed characterization of vesicle mechanics
801 and cell aggregates rheology. *bioRxiv.* 2023.10.19.562871.
- 802 37. Holzapfel, G.A. 2000. Nonlinear solid mechanics: a continuum approach for engineering
803 science. Wiley, Hoboken.
- 804 38. Green, A.E., and W. Zerna. 1968. Theoretical Elasticity. Dover Publications, Mineola.
- 805 39. Naik, S., M. Unni, D. Sinha, S.S. Rajput, P.C. Reddy, E. Kartvelishvily, I. Solomonov, I. Sagi, A.
806 Chatterji, S. Patil, and S. Galande. 2020. Differential tissue stiffness of body column facilitates
807 locomotion of Hydra on solid substrates. *J. Exp. Biol.* 223.
- 808 40. Boot, R.C., A. Roscani, L. van Buren, S. Maity, G.H. Koenderink, and P.E. Boukany. 2023. High-
809 throughput mechanophenotyping of multicellular spheroids using a microfluidic micropipette
810 aspiration chip. *Lab Chip.* 23:1768.
- 811 41. Guevorkian, K., F. Brochard-Wyart, and D. Gonzalez-Rodriguez. 2021. Flow dynamics of 3D
812 multicellular systems into capillaries. In: *Viscoelasticity and Collective Cell Migration: An*

- 813 Interdisciplinary Perspective Across Levels of Organization. Academic Press. pp. 193–223.
- 814 42. Harris, A.R., L. Peter, J. Bellis, B. Baum, A.J. Kabla, and G.T. Charras. 2012. Characterizing the
815 mechanics of cultured cell monolayers. *Proc. Natl. Acad. Sci. U. S. A.* 109:16449–16454.
- 816 43. Brinkmann, F. 2020. Mathematical models and numerical simulation of mechanochemical
817 pattern formation in biological tissues. *PhD thesis, Ruprecht-Karls-Universität.*
- 818 44. Ugural, A.C. 2009. Stresses in beams, plates, and shells, third edition. CRC Press, Boca Raton.
- 819 45. Takeichi, M. 1977. Functional correlation between cell adhesive properties and some cell
820 surface proteins. *J. Cell Biol.* 75:464–474.
- 821 46. Straight, A.F., A. Cheung, J. Limouze, I. Chen, N.J. Westwood, J.R. Sellers, and T.J. Mitchison.
822 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. *Science*
823 *(80-).* 299:1743–1747.
- 824 47. Sato, M., D.P. Theret, L.T. Wheeler, N. Ohshima, and R.M. Nerem. 1990. Application of the
825 Micropipette Technique to the Measurement of Cultured Porcine Aortic Endothelial Cell
826 Viscoelastic Properties. *J. Biomech. Eng.* 112:263–268.
- 827 48. Tsai, M.A., R.S. Frank, and R.E. Waugh. 1993. Passive mechanical behavior of human
828 neutrophils: power-law fluid. *Biophys. J.* 65:2078–2088.
- 829 49. Ruiz-Herrero, T., K. Alessandri, B. V. Gurchenkov, P. Nassoy, and L. Mahadevan. 2017. Organ
830 size control via hydraulically gated oscillations. *Development.* 144:4422–4427.
- 831 50. Lien, J.C., and Y. li Wang. 2021. Cyclic stretching-induced epithelial cell reorientation is driven
832 by microtubule-modulated transverse extension during the relaxation phase. *Sci. Reports*
833 *2021 111.* 11:1–12.
- 834 51. G eremie, L., E. Ilker, M. Bernheim-Dennery, C. Cavaniol, J.L. Viovy, D.M. Vignjevic, J.F. Joanny,
835 and S. Descroix. 2022. Evolution of a confluent gut epithelium under on-chip cyclic stretching.
836 *Phys. Rev. Res.* 4:023032.
- 837 52. Fink, J., N. Carpi, T. Betz, A. B etard, M. Chebah, A. Azioune, M. Bornens, C. Sykes, L. Fetler, D.
838 Cuvelier, and M. Piel. 2011. External forces control mitotic spindle positioning. *Nat. Cell Biol.*
839 *2011 137.* 13:771–778.
- 840 53. Wu, P.H., D.R. Ben Aroush, A. Asnacios, W.C. Chen, M.E. Dokukin, B.L. Doss, P. Durand-Smet,
841 A. Ekpenyong, J. Guck, N. V. Guz, P.A. Janmey, J.S.H. Lee, N.M. Moore, A. Ott, Y.C. Poh, R. Ros,
842 M. Sander, I. Sokolov, J.R. Staunton, N. Wang, G. Whyte, and D. Wirtz. 2018. A comparison of
843 methods to assess cell mechanical properties. *Nat. Methods 2018 157.* 15:491–498.
- 844 54. Veschgini, M., R. Suzuki, S. Kling, H.O. Petersen, B.G. Bergheim, W. Abuillan, P. Linke, S.
845 Kaufmann, M. Burghammer, U. Engel, F. Stein, S.  zbek, T.W. Holstein, and M. Tanaka. 2023.
846 Wnt/ β -catenin signaling induces axial elasticity patterns of Hydra extracellular matrix.
847 *iScience.* 26:106416.

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